Microflora in the honeybee digestive tract: counts, characteristics and sensitivity to veterinary drugs

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Summary — Experiments were carried out to enumerate and characterize the microorganisms in the midgut and rectum of the honeybee. Counts of aerobic microorganisms were distinctly lower than counts of anaerobes (10^4–10^5 viable cells per gram of intestinal contents versus 10^8–10^9 per gram). Total numbers of anaerobic microorganisms were almost identical with counts of anaerobic Gram-positive acidoresistant rods. These bacteria represent the principal groups of microorganisms in the bee digestive tract. Anaerobic and aerobic microorganisms, lactobacilli, coliforms, staphylococci, Bacillus sp, and yeasts were found in all bees. Only one out of 31 isolates (Bifidobacterium asteroides) was identified at the species level. Fluvalinate, fumagillin and nystatin significantly increased mortality of bees. Treated bees kept in cages contained more yeasts than control bees in the beehive. The veterinary drugs tested significantly increased counts of yeasts in comparison with the control.

Apis mellifera / associated microflora / Bifidobacterium / veterinary drugs

INTRODUCTION

The symbiotic microflora of the digestive tract of mature honeybees (Apis mellifera L) consists of Gram-negative, Gram-positive and Gram-variable bacteria, moulds, and under some conditions also yeasts (Gilliam, 1987). The normal microflora is obtained from consumption of pollen, other food, and through contacts with older bees in the colony (Polštěv, 1969; Glinski and Jarosz, 1995). Early work in this field states...
that typical honeybee gut microbes are \textit{Lactobacillus rigidus apis}, \textit{L. constellatus} and \textit{Bacillus influzoides apis} (White, 1921). Also bifidobacteria were often isolated from the honeybee digestive tract (Scardovi and Trovatelli, 1969; Scardovi 1986). Their quantitative significance, however, remains unclear. According to more recent studies, unidentified Gram-variable pleomorphic bacteria and bacteria belonging to the genus \textit{Bacillus} and the family Enterobacteriaceae are the most numerous microbes of the honeybee gut (Gilliam, 1987; Gilliam et al, 1988; Gilliam and Taber, 1991). The intestinal flora of the honeybee is susceptible to various chemotherapeutics and its species composition varies seasonally (Smolska-Szymczewska, 1989). Our knowledge of the microbial ecosystem in the honeybee digestive tract is far from complete. Future research should determine the composition of the microflora of honeybees more exactly, and specifically with host-microbe and microbe-microbe interactions. The aim of our study was to estimate counts of several specific groups of microorganisms in the midgut and rectum of summer and winter honeybees and to isolate, characterize, and identify some typical gut bacteria. We also investigated effects of several veterinary drugs on microbial counts and mortality of bees.

\section*{MATERIAL AND METHODS}

\subsection*{Counting of microorganisms}

Workers of \textit{A mellifera} from the Bee Research Institute (Liběice, Czech Republic) were used for experiments. For the experiment, 25 winter and 25 summer bees were taken from the honeycombs from the same healthy colony. The workers were decapitated and their midgut and rectum were weighed and aseptically transferred into tubes containing sterile Wilkins-Chalgren broth (Oxoid). The tubes were flushed with O$_2$-free CO$_2$ and closed by rubber stoppers. The same broth was used for serial dilutions of all samples. The 0.1-mL aliquots were plated on

\begin{table}[h]
\centering
\caption{Media and cultivation conditions used for enumeration of microorganisms.}
\begin{tabular}{llll}
\hline
Microbial groups & Medium & Length of incubations (h) & Cultivation method & Temperature (\degree C) \\
\hline
Total anaerobes & Wilkins-Chalgren agar & 72 & anaerobic & 37 \\
Gram-positive anaerobic acidoresistant roads & Rogosa agar with cysteine (0.05\% w/v) & 72 & anaerobic & 37 \\
Lactobacilli & Rogosa agar & 72 & microaerophilic & 37 \\
Total aerobes & yeast extract agar with glucose (1\% w/v) & 72 & aerobic & 37 \\
Coliforms & endo agar & 24 & aerobic & 37 \\
Staphylococci & staphylococcus medium & 24 & aerobic & 37 \\
Enterococci & Slanetz-Bartley medium & 48 & aerobic & 37 \\
\textit{Bacillus} spp & nutrient agar & 24 & aerobic & 37 \\
Yeasts & Czapek-Dox agar & 72 & aerobic & 25 \\
Moulds & Czapek-Dox agar & 72 & aerobic & 25 \\
\textit{Pseudomonas} spp & Pseudomonas agar & 48 & aerobic & 37 \\
\hline
\end{tabular}
\end{table}

All media were purchased from Oxoid.
various agar media and incubated until a good microbial growth was noted (table I). Anaerostats (Anaerobic Plus System, Oxoid) and CO₂/H₂ (10/90%) atmosphere were used for anaerobic cultivations. The same equipment filled with a CO₂/O₂/N₂ (10/6/84%) atmosphere was used for microaerophilic cultivation. Colonies were counted and microbial counts expressed as log₁₀ cfu per gram of the gut contents.

Differences among winter and summer bees and among midgut and rectum counts were analyzed using t-test procedure.

Characterization of bacterial isolates

Bacteria grown on the Rogosa agar modified by addition of cysteine hydrochloride (0.05% w/v) were the most numerous groups observed and therefore were presumptive characterized. One-hundred colonies arising on the plates with modified Rogosa agar inoculated with midgut or rectum contents were picked off and examined microscopically after being Gram stained.

Seventeen isolates from the midgut and 14 isolates from the rectum, obtained from plates with Wilkins-Chalgren agar for total anaerobes, were examined. The following characteristics were compared: Gram staining, catalase (Levett, 1991), and oxidase (commercial kit, Lachema Brno, Czech Republic). In order to determine the end-products of glucose fermentation, the bacteria were grown in M10 broth (Caldwell and Bryant, 1966). Lactate was determined by gas chromatography on a column of DB-Wax Mega bore (J & W, USA), acetate and propionate by means of a column with 4% Carbowax 20M on Carbopack B-DA (Supelco, USA). The fructose-6-phosphate phosphoketolase (EC 4 1 2 22) was assayed in all Gram-positive rods producing acetate and lactate (Scardovi, 1986). Phosphoketolase-positive strains were assigned to the genus Bifidobacterium and identified according to their fermentation characteristics (Scardovi, 1986; Biavati et al, 1992; Mitsuoka, 1992) using the API 50 CHL tests (BioMérieux, France). Inoculated plates were incubated anaerobically in CO₂/H₂ atmosphere at 37 °C for 48 h. Other strains were characterized by means of commercial tests produced by Lachema Brno (Anaerotest, Enterotest 1 and 2, Neferntest, Streptotest). Gram-positive, catalase, oxidase and nitrate reductase-negative, nonmotile rods producing mainly lactic acid were assigned to the genus Lactobacillus. The susceptibility of isolates toward metronidazole was tested with antibiotic discs (5 μg), purchased from Oxoid (UK).

Effect of veterinary drugs

Thirty-two laboratory cages (130 x 130 x 60 mm; four cages per group) with 150–200 bees per cage were utilized. Three cages were used for the determination of LT₅₀, one for microbiological analyses. Bees were fed pollen for 10 days and sucrose syrup with or without a drug. Some drugs were evaporated, fumigated or offered in the form of impregnated wood (table II). The applied concentrations were with respect to the number of bees in a cage (100 times lower than 20000 bees, which is considered as mean strong colony). Both temperature (26 °C) and humidity (60%) were controlled. Enumeration of rectum microorganisms was performed weekly, using the methods described above for bees aged 3, 4, 5 and 6 weeks. Control bees were also sampled at the age of 53 days.

For all treated bees a comparison was made at the end of the experiment of the state of construction and the area of constructed cells on the cage foundation with the foundation construction for control bees. 100% indicates fully finished construction of the comb.

Differences among data were analysed using an f-test procedure.

RESULTS AND DISCUSSION

Table III offers counts of main groups of microorganisms in the honeybee digestive tract. Anaerobic and aerobic microorganisms, lactobacilli, coliforms, staphylococci, Bacillus sp, and yeasts were found in all bees. On the other hand, enterococci, pseudomonads and moulds were found only in 6, 8 and 28% of the bees examined, respectively. Their counts varied from 2 to 6 log cfu per gram.

The midgut of winter bees contained more anaerobic microorganisms and yeasts than the midgut of summer bees (P < 0.01). On the contrary, the midgut and rectum of
Table II. Scheme of experiments with veterinary drugs.

<table>
<thead>
<tr>
<th>Trade name of the medicament</th>
<th>Gabon PA 92</th>
<th>M-I</th>
<th>Tylan 200</th>
<th>Nystatin</th>
<th>Fumagilin</th>
<th>Taktivar FUM</th>
<th>Formidol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active substance</td>
<td>Acrinathrin</td>
<td>Fluvalinate</td>
<td>Tylosin</td>
<td>Nystatin</td>
<td>Fumagilin</td>
<td>Amitraz</td>
<td>Formic acid</td>
</tr>
<tr>
<td>Supplier</td>
<td>Bee Research Institute, Dol, Czech Republic</td>
<td>Bee Research Institute, Dol, Czech Republic</td>
<td>Bioveta, Ivanovice, Czech Republic</td>
<td>Sigma</td>
<td>Sanofi, Bratislava, Slovak Republic</td>
<td>Bee Research Institute, Dol, Czech Republic</td>
<td>Bee Research Institute, Dol, Czech Republic</td>
</tr>
<tr>
<td>Method of administration</td>
<td>contact with impregnated wood</td>
<td>contact with impregnated foundation</td>
<td>sugar syrup</td>
<td>sugar syrup</td>
<td>sugar syrup</td>
<td>fumigation</td>
<td>evaporation</td>
</tr>
<tr>
<td>Concentration of the active substance per bee colony</td>
<td>2.4–3.4 mg</td>
<td>0.25%</td>
<td>400 mg</td>
<td>45 mg</td>
<td>140 mg</td>
<td>25 mg</td>
<td>34 mL</td>
</tr>
<tr>
<td>Length of exposure (days)</td>
<td>30</td>
<td>30</td>
<td>21</td>
<td>21</td>
<td>7</td>
<td>1 h</td>
<td>4</td>
</tr>
<tr>
<td>Initial age of bees (days)</td>
<td>11.5</td>
<td>11.5</td>
<td>13.5</td>
<td>13.5</td>
<td>13.5</td>
<td>17.5</td>
<td>17.5</td>
</tr>
</tbody>
</table>

Table III. Microbial counts (log cfu/g) in the midgut and rectum of winter and summer honeybees.

<table>
<thead>
<tr>
<th>Microbiological groups</th>
<th>Winter bees</th>
<th>Summer bees</th>
<th>Winter bees</th>
<th>Summer bees</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total anaerobes</td>
<td>8.62 ± 0.42A*</td>
<td>8.17 ± 0.63B*</td>
<td>9.62 ± 0.43A</td>
<td>9.49 ± 1.05B</td>
</tr>
<tr>
<td>Gram-positive anaerobic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidoresistant rods</td>
<td>8.01 ± 0.55C</td>
<td>7.71 ± 0.70D</td>
<td>9.60 ± 0.51C</td>
<td>9.40 ± 1.37D</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>7.24 ± 0.67D</td>
<td>7.01 ± 0.63E</td>
<td>8.66 ± 0.73D</td>
<td>8.37 ± 1.99E</td>
</tr>
<tr>
<td>Total aerobes</td>
<td>5.63 ± 1.50</td>
<td>5.42 ± 1.04E</td>
<td>5.00 ± 1.36</td>
<td>4.42 ± 1.40E</td>
</tr>
<tr>
<td>Coliforms</td>
<td>2.50 ± 2.63</td>
<td>3.57 ± 1.14</td>
<td>3.77 ± 1.40</td>
<td>3.95 ± 1.70</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>3.22 ± 0.54</td>
<td>3.81 ± 1.03</td>
<td>3.48 ± 0.77</td>
<td>3.75 ± 1.28</td>
</tr>
<tr>
<td>*Bacillus sp</td>
<td>2.44 ± 0.52G*</td>
<td>3.72 ± 0.69*</td>
<td>3.59 ± 0.87G</td>
<td>3.45 ± 0.60</td>
</tr>
<tr>
<td>Yeasts</td>
<td>5.48 ± 1.41H*</td>
<td>3.92 ± 1.03*</td>
<td>3.80 ± 1.02H</td>
<td>3.41 ± 0.90</td>
</tr>
</tbody>
</table>

* Differences among winter and summer samples (P < 0.01).
A–H Capital letters indicate differences among the midgut and rectum data (P < 0.01). All counts are means from 25 determinations.
Table IV. Biochemical characteristic of pure bacterial strains isolated from the midgut (Nos 1–17) and rectum (Nos 18–30).

| Mannitol | Glyceral | Sorbitol | Inositol | Adonit | Dulcit | Xylose | Fructose | Galactose | Rhamnose | Maltose | Lactose | Sucrose | Trehalose | Cellobiose | Melezitose | Raffinose | Inulin | Arginin | dihydrolase | Ornithine | Decarboxylase | Phenylalanin | deaminase | Hydrogen | sulfide | Urease | Phosphatase | ONPG test | NaCl-esculin | Bile-esculin | Simons | citrate | Nitrate | Catalase | Products from glucose  |
|----------|----------|----------|----------|--------|--------|--------|----------|----------|----------|---------|---------|---------|---------|----------|-----------|------------|---------|--------|--------|-----------|----------|-------------|-------------|---------|---------|---------|----------|-----------------|
| -        | +        | +        | -        | +      | +      | +      | +        | +        | +        | +       | -       | +       | -       | +        | +         | +          | -       | +      | -       | -         | +        | -            | -            | +       | -       | +       | +         | LA AL LA LA L AL AL AL L L NT L AL P A NT L L LA AL LA AL LA LA L NT L LA LA L |
|          |          |          |          |        |        |        |          |          |          |         |         |         |         |          |           |            |          |        |        |           |          |              |              |         |        |         |           | Morphology: R R R C R R R R R R R R R R R R R R R R |

* A, acetate; L, lactate; NT, not tested; P, propionate; ^c, coccus; R, rod; "w, weak reaction.  
All strains fermented glucose and salicin. No strain fermented arabinose and sodium malonate. All strains hydrolyzed esculin. The following reactions were negative among all the strains tested: Lysine decarboxylase, Voges-Proskauer, oxidase, and lipase (tween 80). All strains were gram positive, nonmotile, nonsporulating and resistant to metronidazole (5 μg).
summer bees harboured more coliforms and staphylococci. Counts of aerobes were distinctly lower than counts of anaerobes. Numbers of aerobes varied more than the numbers of anaerobes. Total numbers of anaerobic microorganisms were practically identical with bacterial counts found on Rogosa agar with cysteine under anaerobic atmosphere. These bacteria can be simply characterized as anaerobic Gram-positive acidoresistant rods. The classification was confirmed by Gram stain and by the ability to grow in the presence of acetic acid (pH 5.4), which is routinely added to the Rogosa medium. Counts of lactobacilli were ca 7–8 log cfu per gram of midgut and rectum contents. Counts of the most numerous groups of microorganisms were significantly \((P < 0.01)\) higher in the rectum in comparison to midgut counts.

Only one isolate \((Bifidobacterium asteroides)\) was identified at the species level. Other isolates were Gram-positive rods (24 strains), or cocci (two strains) fermenting glucose and salicin and able to hydrolyze aesculin. All strains were resistant to metronidazole. Attempts to identify lactobacilli (strain Nos 11, 14, 18 and 19) at the species level failed. Biochemical characteristics of isolates are shown in table IV.

In previous reports, the principal bacteria of the bee digestive tract were described as Gram-variable pleomorphic rods with uncertain taxonomy (Gilliam, 1987; Gilliam et al, 1988; Gilliam and Taber, 1991). In our experiments, the most numerous microorganisms were Gram-positive bacterial rods of rather regular shape. Several authors mentioned the presence of bifidobacteria among organisms isolated from the bee gut (Scardovi and Trovatelli, 1969; Scardovi, 1986; Biavati et al, 1992). In our opinion, bifidobacteria belong to typical but not dominant bacterial species in the bee gut. The most numerous organisms seem to be Gram-positive rods related to the genus \(Lactobacillus\).

Table V summarizes the results of the effect of some veterinary drugs on the bee gut microflora. Again, anaerobic bacteria

<table>
<thead>
<tr>
<th>Drug</th>
<th>Total anaerobes</th>
<th>Gram-positive anaerobic acidoresistant rods</th>
<th>Lactobacilli</th>
<th>Total aerobes</th>
<th>Yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gabon PA 92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(acrinathrin)</td>
<td>10.01 ± 1.22</td>
<td>10.00 ± 1.30</td>
<td>9.50 ± 0.89</td>
<td>5.46 ± 0.34</td>
<td>6.67 ± 0.25*</td>
</tr>
<tr>
<td>M-1 (fluvalinate)</td>
<td>10.00 ± 1.00</td>
<td>9.92 ± 1.25</td>
<td>9.13 ± 0.61</td>
<td>3.98 ± 0.63</td>
<td>6.99 ± 0.88*</td>
</tr>
<tr>
<td>Tylan 200 (tylosin)</td>
<td>9.50 ± 0.78</td>
<td>9.68 ± 0.99</td>
<td>8.84 ± 0.53</td>
<td>5.68 ± 0.30</td>
<td>6.84 ± 0.53*</td>
</tr>
<tr>
<td>Nystatin</td>
<td>9.98 ± 1.00</td>
<td>9.87 ± 0.85</td>
<td>9.04 ± 0.56</td>
<td>5.26 ± 0.59</td>
<td>7.40 ± 1.11*</td>
</tr>
<tr>
<td>Fumagillin</td>
<td>10.02 ± 1.21</td>
<td>10.06 ± 0.91</td>
<td>9.36 ± 0.58</td>
<td>4.96 ± 0.72</td>
<td>6.79 ± 0.56*</td>
</tr>
<tr>
<td>Taktivar FUM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(amitraz)</td>
<td>10.09 ± 0.92</td>
<td>9.91 ± 0.87</td>
<td>9.27 ± 0.71</td>
<td>4.56 ± 0.99</td>
<td>7.87 ± 1.13*</td>
</tr>
<tr>
<td>Formidol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(formic acid)</td>
<td>9.99 ± 0.94</td>
<td>9.99 ± 1.19</td>
<td>9.36 ± 0.78</td>
<td>NT*</td>
<td>NT</td>
</tr>
<tr>
<td>Control (cages)</td>
<td>9.97 ± 0.98</td>
<td>9.95 ± 1.00</td>
<td>9.24 ± 0.83</td>
<td>4.88 ± 1.13</td>
<td>6.10 ± 1.20</td>
</tr>
<tr>
<td>Control (beehive)</td>
<td>9.96 ± 0.67</td>
<td>10.05 ± 0.82</td>
<td>9.07 ± 0.27</td>
<td>5.24 ± 1.32</td>
<td>4.32 ± 0.99</td>
</tr>
</tbody>
</table>

*NT, not tested. Values are means \((\log_{10})\) ± SD per gram for quadruplicate determination.

* Differences among treated and control (beehive) bees \((P < 0.01)\).
were the most numerous microorganisms (9–10 log cfu/g). Treated bees kept in cages contained more yeasts and yeast-like organisms than control bees in the beehive (P < 0.01). Gilliam et al (1977) and Gilliam (1987) found high counts of yeasts in stressed bees kept in cages, fed a diet deficient in some nutrients or fed a diet contaminated with pesticides. Counts of total anaerobes, anaerobic Gram-positive acidoresistant rods and lactobacilli were lower, and those of aerobes were higher in tylosin-fed bees than in bees of other groups. Fluvalinate M-1 decreased counts of aerobic microorganisms and amitraze (Taktivar Fum) caused proliferation of yeasts. But all these differences were not significant. Fumagillin and nystatin had generally little influence on microbial counts (table V). Smolska-Szymcewska (1989) observed lower counts of coliform bacteria in bees fed fumagillin. Our results are in line with findings of Gilliam (1986) and Prabucki and Górski (1987) who noticed an increase in numbers of yeasts caused by fumagillin.

Veterinary drugs had a harmful effect on bees as evidenced by mortality data (table VI). Since the variability in mortality data was high, only the effect of fluvalinate, fumagillin, and nystatin was significant (P < 0.05). Subsequently, lower production of cage foundation was observed. Aerobic bacteria Bacillus cereus, Acro- mobacter sp, and Pseudomonas fluorescens degrade fluvalinate in vitro (Maloney et al, 1988). These bacteria are present in the honeybee digestive tract, but their numbers, however, are very low as follows from our results. In conclusion, veterinary drugs for the honeybees tested in this study more or less changed the composition of the honeybee gut microflora and often increased the mortality of bees. Previous studies on interactions of drugs with the honeybee gut microbes utilized aerobic bacteria, eg, bacilli (Drobníková and Baciček, 1982). These bacteria, however, were not dominant organisms of the bee digestive tract in our study.

**ACKNOWLEDGMENT**

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Les médicaments vétérinaires ont eu un effet défavorable sur l’abeille, comme le montre les taux de mortalité (tableau VI). Le fluvalinate, la fumagilline et la nystatine appliquées aux abeilles encagées durant 3 semaines ont augmenté significativement la mortalité (p < 0,05). Les abeilles encagées contenaient plus de levures que les témoins ; le même effet a été observé après application des médicaments vétérinaires (tableau V).

Des études précédentes sur l’interaction entre les médicaments vétérinaires et les microorganismes intestinaux, les bactéries aérobies, comme par exemple B cereus, ont été utilisées en majorité. Les médicaments vétérinaires testés ont eu un effet réduit sur la composition de la microflore intestinale.


Fast alle anaeroben Bakterien waren anaerobe, grampositive und säureresistente Stäbchen. Sie bilden demnach die Hauptgruppe der Mikroorganismen im Verdauungsstrakt der Bienen (10⁹/g). Die Anzahl der Laktobazillen betrug etwa 10⁷–10⁸/g (Tabelle III). Bei allen Bienen wurden anaerobe und aerobic Bakterien, Laktobazillen, coliforme Bakterien, Staphylokokken, Bacillus sp und Hefepilze gefunden. Enterokokken, Pseudomonaden und Schimmelpilze

*Apis mellifera* / assoziierte Microflora / *Bifidobacterium* / Veterinärarzneimittel

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